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PHOTORECEPTOR REDOX STATE MONITORED *IN VIVO* BY TRANSMISSION AND FLUORESCENCE MICROSPECTROPHOTOMETRY IN BLOWFLY COMPOUND EYES

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Abstract—The transmission and fluorescence of the compound eye of living, intact blowflies *Calliphora erythrocephala*, mutant *chalky*, were studied microspectrophotometrically. Transmission spectra were recorded under four conditions. The fly was either in the normal air environment or in a nitrogen atmosphere, and in both cases the investigated eye was adapted to red and blue light, respectively.

The absorbance difference spectra obtained from the two chromatic adapted conditions showed the clear characteristics of the main visual pigment; the difference spectra for the air and the N_2 case were virtually identical.

The absorbance difference spectrum obtained from the air vs N_2 case was very similar to the redox difference spectrum of the pigments in the mitochondrial chain. The redox difference spectra obtained for the two photosteady states were essentially the same.

The fluorescence emission spectra induced by UV and blue excitation were measured with the fly in air and in a nitrogen atmosphere, respectively. The UV-induced blue emission increased under hypoxia, whereas the blue-induced green emission dropped. The changes are typical for a reduction of mitochondrial NADH and flavoproteins, respectively.

The transmission and fluorescence measurements corroborate each other and demonstrate mitochondrial activity in photoreceptors *in vivo* and non-invasively.

Photoreceptor metabolism Blowfly Hypoxia Microspectrophotometry Microspectrofluorometry Visual Pigments Mitochondrial pigments

INTRODUCTION

Microspectrophotometry of photoreceptor cells has become an almost routine technique in vision research since the first studies on visual pigments *in situ* were started a quarter of a century ago (rev. Liebman, 1972). Probably due to their primary role in vision the visual pigments have received the major attention of the spectroscopists. Later on, the photostable (e.g. screening) pigments, such as those in oil droplets have attracted extensive interest (e.g. Muntz, 1972). On the other hand, the pigments of the mitochondrial respiratory chain, the third pigment class of importance for photoreceptors, have gained less interest.

Liebman (1969) presented a record from the ellipsoid, i.e. the mitochondrial region, of a cone cell in *Necturus*. The record shows the presence of several types of cytochrome oxidative enzymes in the reduced state. Liebman stated: "It is not known if a metabolic response is initiated in the mitochondria by light absorbed in the photoreceptor outer segment nor is it apparent what time course such a response would take. The answer to these questions might be provided by microphotometry." Indeed, recently we observed (Stavenga and Tinbergen, 1983), in the course of our *in vivo* microspectrofluorometrical studies on the visual pigments in blowfly eyes, transient fluorescence changes, with a time course in the order of seconds, and hypothesized that these changes originated in light-induced redox changes in the mitochondrial respiratory chain. The evidence was mainly obtained from measurements on the dependence of the phenomenon on light intensity and oxygenation. We therefore decided to characterize the spectral properties of the pigments involved by both transmission and fluorescence microspectrophotometry.

MATERIALS AND METHODS

The blowfly *Calliphora erythrocephala*, white eyed mutant *chalky*, was investigated. This mu-

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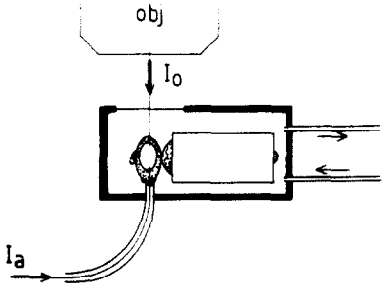

Fig. 1. Diagram of housing with immobilized intact fly. Arrows indicate gas flow for application of air or N_2 atmosphere. In the transmission measurements illumination I_a was applied via a small light guide at the ventral side of the eye. In the fluorescence measurements illumination I_0 was applied via the objective of the microspectrophotometer.

tant lacks the screening pigments, which exist normally, i.e. in the wild type fly, in both the pigment cells and the photoreceptor cells, but in all other aspects, the retinal properties of mutant and wild type are considered identical.

The fly was immobilized with wax (and remained thus intact and alive; when occasionally a drop of sugar water is supplied, experiments with one and the same fly then can easily be extended over several days). The fly was subsequently mounted in a housing on a Leitz Universal stage. A window, consisting of a sealed-in cover slip, allowed the optical measurements (see Fig. 1). The composition of the atmosphere in the housing was changed through tubes connected to a selected gas cylinder. The atmospheres were always at room temperature.

For the transmission measurements a plastic light guide (diameter 0.5 mm) was introduced through a tightly fitting hole in the wall of the housing and its tip was positioned ventrally to the compound eye (Fig. 1). The other end received light from a 450 W Xe-arc filtered spectrally by an Oriel grating monochromomator. The objective of the microspectrophotometer (MSP) sampled light from *c*.

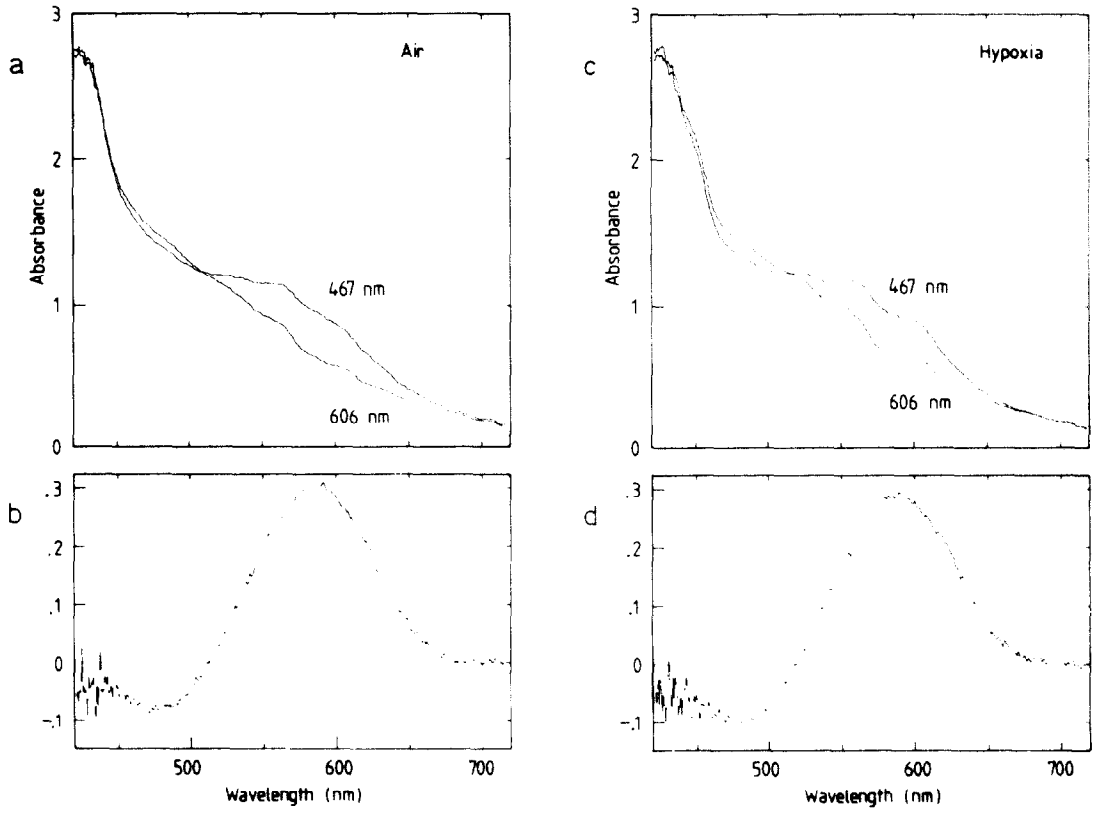

Fig. 2. Absorbance spectra of the photoreceptor layer of the blowfly eye and their absorbance difference spectra. (a) Absorbance spectra of the eye adapted to blue (467 nm) and red (606 nm) light, respectively, when placed in a normal (air) atmosphere. (b) Absorbance difference between the two spectra of *a*. (c) Absorbance spectra of the eye adapted to blue (467 nm) and red (606 nm) light under hypoxia conditions induced by a N_2 atmosphere. (d) Absorbance difference between the spectra of *c*. Absorbance was calculated as the $\log_{10}$ of the ratio between the light signal obtained from the light guide directly and that with the blowfly eye in the light beam. However, the ordinate values were lowered by approximately 3 log units for clarity's sake.

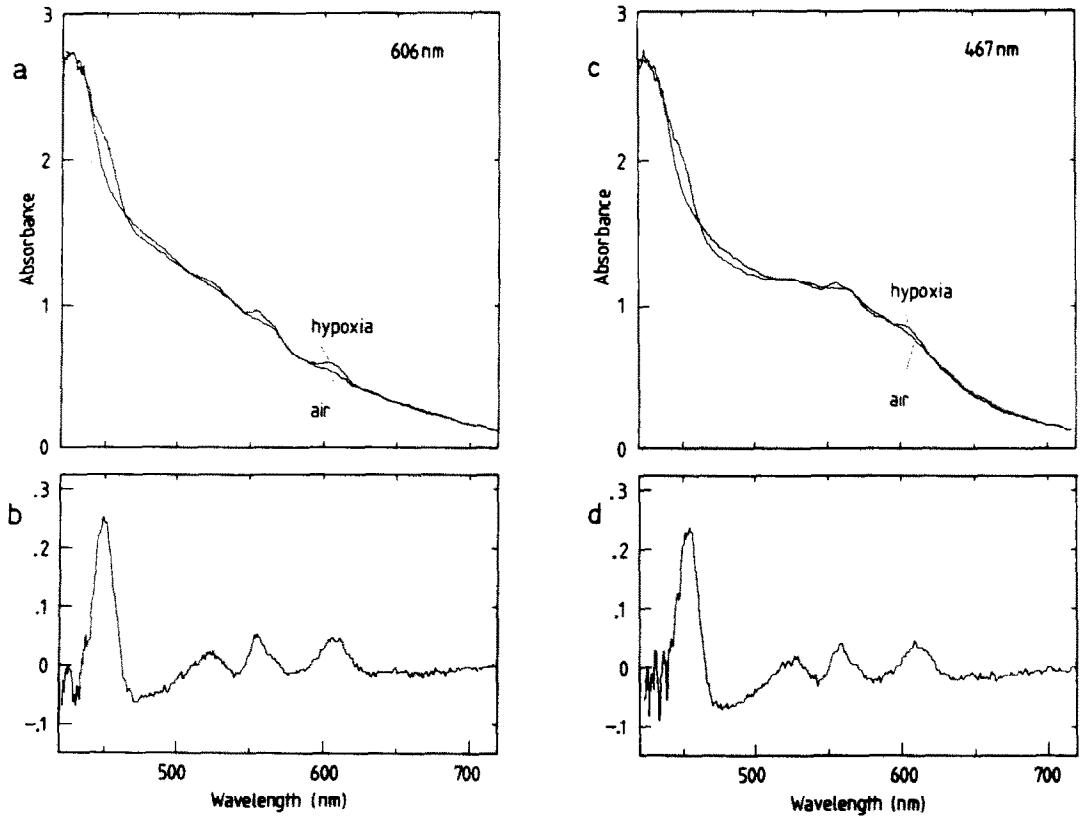

Fig. 3. Absorbance spectra of the photoreceptor layer of the blowfly eye and their absorbance difference spectra. (a) Absorbance spectra of the red (606 nm) adapted eye under normal (air) and hypoxia conditions. (b) Absorbance difference between the spectra of *a*. (c) Absorbance spectra of the blue (467 nm) adapted eye under normal (air) and hypoxia conditions. (d) Absorbance difference between the spectra of *c*.

200 facets in the dorsal part of the eye, i.e. light which has been transmitted (scattered) through the photoreceptor layer.

In the fluorescence measurements the excitation light was delivered by the objective, and the induced emission was similarly collected (epi-fluorescence).

The presented spectra (Figs 2 and 3) were from one and the same fly. For each spectrum 10 transmission runs were averaged. Each run took 15 sec. 2 min were taken in order to achieve a stable hypoxia state.

The MSP is a Leitz Orthoplan equipped with a Ploemopak illuminator and a modified Leitz Compact photometer head. The modification is a motorized interference wedge (Schott Veril S200, 400–700 nm), in front of the Hamamatsu R928 photomultiplier, thus enabling a spectral analysis of the emission beam. The objective was a Leitz NPL 10,0.20.

The excitation light was supplied by a 75W Xe-arc filtered spectrally by interference filters.

In the experiments with UV-excitation broad band illumination (Balzers K36) was applied, and the emission was measured above 400 nm (Leitz cube A). At blue-excitation narrow band light of 477 nm (Schott DAL) was applied, and emission was measured above 510 nm (Leitz cube H2). The illumination procedures as well as the spectral scans were controlled by a microcomputer (Data General MP200), which also sampled the photomultiplier signals. Processing of the spectra was performed as follows. Absorbance spectra were calculated from the transmission measurements as usual.

Emission spectra were calculated by relating the measured emissions to that of a known source, i.e. a halogen lamp (12 V, 100 W; Osram) run at 7.0 A, which is equivalent to Planck's black body at 2700 K. The spectra are given as number of quanta emitted per unit wavelength and are normalized to the peak value.

It should be known that the main visual pigment of blowflies is a xanthopsin (X, a visual pigment having 3-hydroxy retinal as chromophore; see Vogt, 1983; Vogt and Kirschfeld, 1984), which absorbs maximally in the blue-

green. Upon light absorption this state converts into a thermostable metaxanthopsin (M) state which absorbs maximally in the orange. M can be photoreconverted into X. Prolonged red light establishes a photosteady state with ≈100% xanthopsin, whereas under blue illumination the photoresteady state can lead to ≈20% X and 80% M (see e.g. Hamdorf, 1979).

RESULTS

The transmission of the photoreceptor layer of the white eyed blowfly mutant *chalky* was measured in the normal situation, i.e. in air in two photosteady states, established by 606 and 467 nm, respectively, yielding the absorbance spectra of Fig. 2a.

The absorbance difference spectrum (Fig. 2b) is the clear characteristic of the main blowfly visual pigment (see e.g. Stavenga and Schwemer, 1984).

Transmission measurements during application of nitrogen yielded the absorbance spectra presented in Fig. 2c. Since the resulting difference spectrum (Fig. 2d) is virtually identical to that measured in air (Fig. 2b) it appears that the visual pigment is not affected by hypoxia.

Figures 3a and 3c present the same spectra as Figs 2a and 2c, but they are now combined differently, namely Figs 3a and 3c represent the cases for air and hypoxia, respectively, with the visual pigment in each case being in one and the same photosteady state. Figure 3a is the case for the photosteady state established by 606 nm (high xanthopsin) and Fig. 3c that at 467 nm (low xanthopsin). The corresponding difference spectra (Fig. 3b and 3d) are virtually identical and are quite reminiscent to the redox difference spectrum of the respiratory pigments in the mitochondrial chain (Chance and Williams, 1956).

Measurement of the fluorescence from the white eyed mutant blowfly yielded the spectra of Fig. 4. Clearly upon hypoxia the UV-induced emission increases (Fig. 4a). On the other hand, blue-induced emission decreases upon hypoxia. We note that the intense excitation light established photosteady states with low xanthopsin contents well before the spectral recordings were started.

DISCUSSION

The spectral properties of fly visual pigment measured *in vivo*, with the fly in air and under

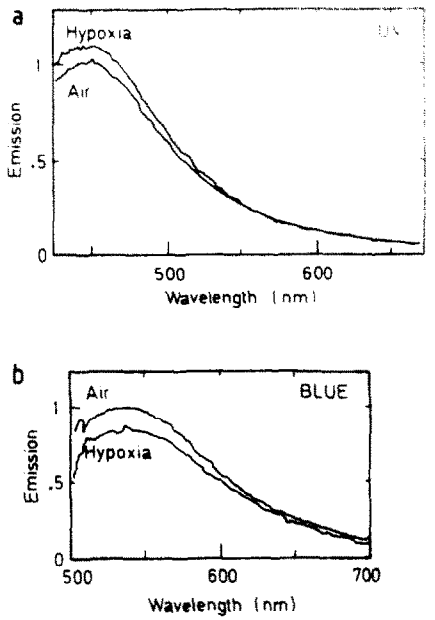

Fig. 4. Emission spectra of the blowfly compound eye, measured in air and under hypoxia, respectively. (a) UV-induced emission spectra. (b) Blue-induced emission spectra.

hypoxia, respectively, are identical in both cases (Fig. 2) and are in close agreement with previous measurements with different techniques (see e.g. Stavenga, 1976; Schwemer, 1983).

On the other hand, we found substantial absorption changes by retinal pigments upon hypoxia, which we attributed to the respiratory pigments of the mitochondria, which exist abundantly within the photoreceptor cells (e.g. Boschek, 1971; Trujillo-Cenóz, 1972). The difference spectra of Fig. 3 are very similar to those of mitochondria (e.g. Chance and Williams, 1956). The peaks appearing at 605, 555 and 445 nm are ascribed to cytochromes a, a_3 and c, c_1 and a, a_3, respectively, whereas the trough at 480 nm is attributed to flavoproteins (see also e.g. Piantidosi and Jobsis-Vandervliet, 1984). The obtained spectra varied only very slightly with eye location. Different flies also yielded insignificant variations.

We did not extend our absorption measurements into the UV. Presumably we then would have encountered a substantial absorbance increase upon hypoxia due to the reduction of NAD^+ to NADH as occurs with isolated mitochondria (Chance and Williams, 1956). This expectation seems reasonable as we found a clear sign of such a reduction in our fluorescence measurements, which show an increase in UV-induced emission (Fig. 4a). The latter phenomenon, as well as the decrease in

blue-induced green emission (Fig. 4b), which represents the reduction of flavoproteins upon hypoxia, were both investigated in detail by Chance and co-workers in isolated mitochondria (Chance and Schoener, 1966; Scholz *et al.*, 1969). Our *in vivo* spectra accord well with these studies.

We note here that substantial concentrations of cytochrome have been discovered in the ellipsosomes of fish photoreceptors (MacNichol *et al.*, 1978; Avery and Bowmaker, 1982). Also, spectra of presumably respiratory hemoproteins, measured from primate foveas, were reported by Snodderly *et al.* (1984).

The present work reinforces our hypothesis (Stavenga and Tinbergen, 1983) that redox changes of the respiratory pigments in fly photoreceptors can be measured in completely intact, living animals. The reported rapid fluorescence changes occurring upon bright light flashes (Stavenga and Tinbergen, 1983) were interpreted as resulting from transient changes in mitochondrial respiratory activity. Having characterized the spectral properties of the pigments involved we have embarked upon a further analysis of mitochondrial activity in fly photoreceptors.

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